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Increased number of $CD4^-$ $CD8^+$ MHC class II-specific T cells in MHC class II-deficient mice

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SUMMARY

Targeted disruption of the Aβ-encoding gene of $H2^b$ mice abolishes major histocompatibility complex (MHC) class II expression and results in a failure to develop $CD4^+$ 8^- T cells. Besides this major effect, the lack of class II expression affects the level of T-cell receptor (TCR) and CD4 expression on differentiating thymocytes. Moreover, there is no class II-mediated negative selection of thymocytes. All this could result in TCR repertoire changes of the $CD4^-$ 8^+ T-cell subpopulation, which apparently develops normally in these mice. To test this hypothesis, the class II reactivity of $CD4^-$ 8^+ T cells from class II-deficient (class II^0) mice was analysed. It was found that $CD4^-$ 8^+ T cells from class II^0 but not from class II-expressing mice developed a significant level of cytotoxicity against class II-expressing target cells. These results demonstrate an influence of MHC class II molecules on the TCR repertoire of $CD4^-$ 8^+ T cells.

INTRODUCTION

Neonatal blockade of class II major histocompatibility complex (MHC) molecules with monoclonal antibodies (mAb), as well as the lack of class II expression due to targeted disruption of the Aβ-encoding gene, interferes with positive selection of $CD4^+$ 8^- T cells.[1–6] The lack of $CD4^+$ 8^- T cells in class II-deficient mice (class^0), homozygous for targeted mutation of Aβ, results in abrogated T-cell-dependent antibody production and complete absence of germinal centres.[3,4] On the other hand, $CD4^-$ 8^+ T cells develop normally in such mice.[3–6] Their cytotoxic functions are preserved, as these mice develop an almost normal cytotoxic T lymphocyte (CTL) response against the influenza A virus nucleoprotein.[7] However, significant impairment of memory responses was observed when these mice were not kept under specific pathogen-free (SPF) conditions.[6,7] Thus, class II^0 mice have been extremely useful for investigating the role of CD4+ T cells in the development of efficient immune responses *in vivo*.

Class II^0 mice are also an ideal tool for studies on the influence of class II molecules on the development of $CD4^-$ 8^+ T cells. Both positive and negative selection of $CD4^-8^+$ T cells can be affected by the lack of class II expression.[8] At the double-positive stage of development, a one- to twofold increase of CD4 expression and a two- to threefold increase of T-cell receptor (TCR) expression have been observed.[8] This can result in different binding avidities during cell–cell contact and a different quantity and/or quality of signals received by the differentiating cells. Such changes were shown to play a role in positive selection of $CD4^-$ 8^+ T cells.[8] Moreover, the lack of negative selection of precursor T cells expressing receptor with a high affinity for class II molecules can influence the TCR repertoire of mature $CD4^-$ 8^+ T cells.[9]

In the present study, we analysed the TCR repertoire of $CD4^-$ 8^+ T cells in class II^0 mice by testing their capacity to recognize allogeneic MHC class II molecules. We demonstrated a significant increase in the number of class II-specific, $CD4^-$ 8^+ cytotoxic T cells in the periphery of MHC class II-deficient mice compared with normal control mice.

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Abbreviations: class-II^0, class II-deficient; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; PE, phycoerythrin.

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MATERIALS AND METHODS

Animals

Mice, kept under SPF conditions in the animal facility at the Max-Planck-Institut (Tübingen, Germany), were used at 8–24 weeks of age. Class II^0 mice, with a homozygous mutation in the gene for Aβ and abrogated MHC class II expression, and heterozygous class $II^{0/+}$ mice, with normal expression of MHC class II molecules, were originally generated by Cosgrove *et al.*[3] and were generously provided by Dr P. Overath. (Max-Planck-Institüt für Biologie, Abteilung Membranbiochemie, Tübingen). They were used at 5–8 months of age. $129^{-/-}$ mice, with a homozygous mutation in the gene for β_2-microglobulin (β_2M) and diminished MHC class I expression, and $129^{+/+}$ mice, with normal expression of the β_2M gene, were originally generated

Table 1. MHC haplotypes of mice used in this study[25]

Mouse strains	H-2 haplotypes	Expressed MHC molecules K	A	E	D	L
C3H	k	k	k	k	k	
B10.BR	k	k	k	k	k	
B6	b	b	b		b	
129	b	b	b		b	
DBA/2	d	d	d	d	d	d
$\beta_2M^{-/-}$	b		b			
class II^0	b	b			b	
B10.GD	g2	d	d		b	
B10.HTG	g	d	d	d	b	
B10.A(2R)	h2	k	k	k	b	
B10.A(4R)	h4	k	k		b	
B10.A(5R)	i5	b	b	k/b	d	d
B10.D2(R107)	i7	b	b		d	d
bm12	bm12	b	bm12		b	
B10.A	a	k	k	k	d	d
B10.AQR	y1	q	k	k	d	d

by Zijlstra *et al.*,[10] and were also provided by Dr P. Overath. MHC-deficient mice and their normal littermates were kept under identical, SPF conditions in a laminar flow unit. All mice received autoclaved food and water. The haplotypes of the mice used in this study are listed in Table 1.

Depletion of $CD4^+$ T-cell subpopulations

$CD4^+$ T cells were depleted using specific mAb. Briefly, the spleen cells were resuspended in medium containing anti-CD4 mAb (RL172.4),[11] with a final concentration of 50% of the hybridoma cell culture supernatant. The cells were incubated for 15 min on ice. After addition of 5% rabbit low toxicity serum and 5% guinea-pig complement the incubation was continued for 30 min at 37°.

Flow cytometric analysis of T-cell subpopulations

Aliquots of unfractionated spleen cells, spleen cells depleted of $CD4^+ 8^-$ T cells and cultures from mixed lymphocyte cultures (MLC), were stained for two-colour analysis. Monoclonal antibodies used for staining were anti-CD8 (YTS169.4), fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-CD4 (YTS191.1), PE-conjugated anti-$\alpha\beta$ TCR (H57-597), FITC-conjugated and anti-$\gamma\delta$ TCR (GL-3), FITC-conjugated goat anti-mouse Ig (all from Medac, Hamburg, Germany). For Thy-1.2 detection, mAb HO13.4 was conjugated with FITC. Stained cells were then analysed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA).

In vitro *induction of CTL against alloantigens*

MLC of 3×10^6 splenic responder cells, or splenic cells depleted of $CD4^+ 8^-$ T cells, and 3×10^6 irradiated (2000 rads) splenic stimulator cells, were set up in 2 ml α-modified Eagle's medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol. Rat concanavalin A (Con A) supernatant (4–5%) was added as a source of lymphokines. The ^{51}Cr-release assay was performed on day 5, with ^{51}Cr-labelled Con A blasts or lipopolysaccharide (LPS) blasts as target cells. All tests were done in triplicate. Standard deviations from the mean values for percentage specific ^{51}Cr release did not exceed 5%. The results were evaluated for statistical significance by the Student's *t*-test. Specific ^{51}Cr release = (experimental − spontaneous release)/(maximum − spontaneous release) × 100.

RESULTS

General remarks

Although the large majority of $CD4^- 8^+$ T cells are specific for antigen presented by MHC class I, some $CD4^- 8^+$ T cells react against allogeneic MHC class II molecules.[12] The lack of CD4 class II and/or TCR class II interaction in the thymus of class II^0 mice could affect T-cell development at various stages. In the present report, we analysed the effect of abrogated MHC class II expression on the number of mature $CD4^- 8^+$, class II-specific T cells in these mice. We stimulated unfractionated spleen cells or spleen cells depleted of $CD4^+ 8^-$ T cells from class II^0 mice and class II-expressing control mice with allogeneic stimulators. After 5 days of culture, the levels of cytolytic activities that developed in these cultures were tested against different allogeneic, class I- and/or class II-expressing targets cells.

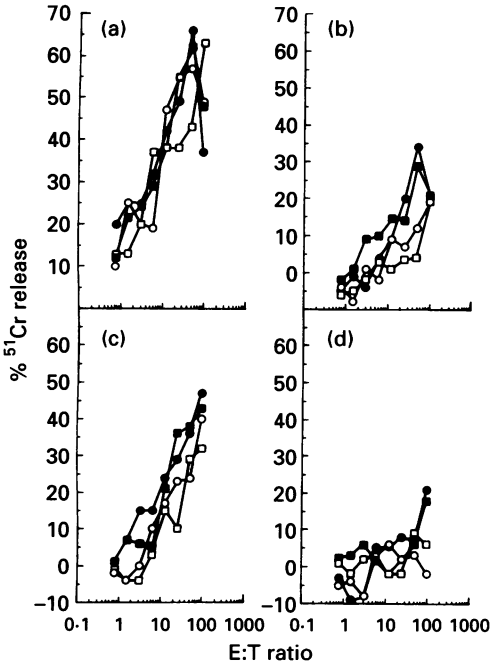

Figure 1. Generation of CTL from spleen cells of mice homozygous (class II^0, closed symbols), or heterozygous (class $II^{0/+}$, open symbols) for disruptive mutation of the Aβ-encoding gene stimulated with irradiated C3H spleen cells. Responder cells were treated with anti-CD4 and complement (circles) or complement only (squares) before the culture period. Cytotoxicity was tested against B10.A Con A blasts (a), B10.A LPS blasts (b), B10.AQR Con A blasts (c) and B10.AQR LPS blasts (d). E:T, effector to target ratio.

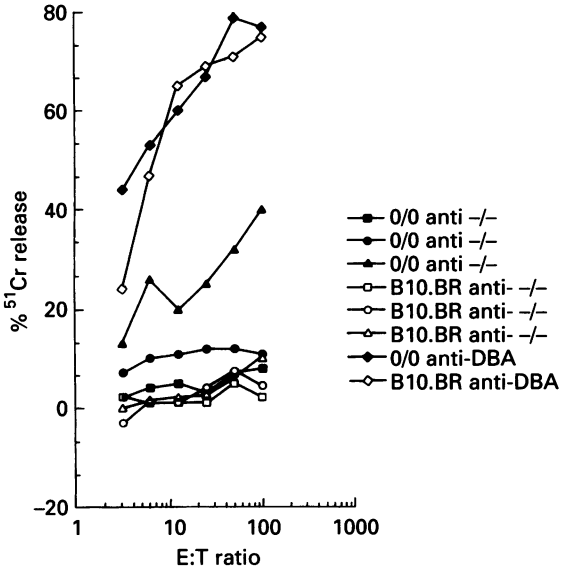

Figure 2. Generation of CTL from spleen cells of mice homozygous for disruptive mutation of the Aβ-encoding gene (class II^0, closed symbols) and B10.BR mice (open symbols). Both types of responders were treated with anti-CD4 and complement before the culture period. Responder cells were stimulated with spleen cells from β_2M-deficient mice (class I-deficient, $\beta_2M^{-/-}$) or DBA/2. Cytotoxicity was tested against P815 (◆, ◇), $\beta_2M^{-/-}$ LPS blasts (▲, △), $\beta_2M^{-/-}$ Con A blasts (■, □) and Con A blasts from normal class I-expressing mice of the same background as $\beta_2M^{-/-}$ mice (●, ○).

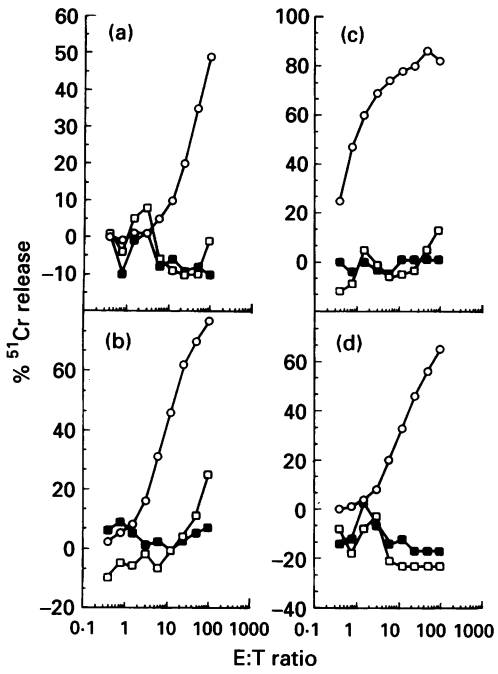

Figure 3. Generation of CTL from spleen cells of various mouse strains depleted of CD4$^+$ T cells. (a) B10.D2(R107) spleen cells were stimulated with irradiated spleen cells of B10.A(5R) mice. Cytotoxicity was tested on B10.A(5R) LPS (□) and Con A (■) blasts. (b) B10.A(4R) spleen cells were stimulated with irradiated spleen cells of B10.A(2R) mice. Cytotoxicity was tested on B10.A(2R) LPS (□) and Con A (■) blasts. (c) B10.GD spleen cells were stimulated with irradiated spleen cells of B10.HTG mice. Cytotoxicity was tested on B10.HTG LPS (□) and Con A (■) blasts. (d) B6 spleen cells were stimulated with irradiated spleen cells of bm12 mice. Cytotoxicity was tested on bm12 LPS (□) and Con A (■) blasts. In all the experiments, fractions of the responder cells were also stimulated with irradiated allogeneic DBA/2 spleen cells, and the cytotoxicity was tested against P815 target cells (○).

CD4$^-$ 8$^+$, MHC class II-specific, cytotoxic T cells are not detectable in MLC with allogeneic MHC class I- and class II-expressing stimulator cells

Unfractionated spleen cells or CD4-depleted spleen cells from mice homozygous or heterozygous for targeted Aβ gene disruption were used as responders in MLC. Spleen cells from C3H mice, which differ from the responders in both MHC class I and class II molecules, were used as stimulators (see Table 1 for the MHC haplotypes of the mouse strains used in this study). After 5 days, cytotoxicity was tested against B10.A and B10.AQR Con A and LPS blasts (Fig. 1; Con A blasts express only class I molecules, while LPS blasts express both class I and class II molecules). All the effectors reacted against Con A and LPS blasts from B10.A mice, which shared class I and class II molecules with the stimulators (Fig. 1a, b). On the other hand, only low levels of cytolysis of B10.AQR LPS blasts was observed. B10.AQR mice expressed the same MHC class II molecules as the stimulators but differed in MHC class I (Fig. 1d). The relatively high cytotoxicity seen with B10.AQR Con A blasts (Fig. 1c) was most probably due to cross-recognition, which is often found when MLC are carried out in the presence of added growth factors. In summary, although normal class I-specific cytotoxicity was observed, class II-specific cytotoxicity was not detected in these cultures.

Increased numbers of CD4$^-$ 8$^+$, class II-specific cytotoxic T cells in MHC class II-deficient mice

Not all T-cell clones specific for allogeneic stimulators develop in primary MLC. Usually, a few clones dominate the cultures and mask the presence of other clones.[13–15] This is expected particularly of CD8+ T cells, which have a high capacity for rapid expansion in the presence of exogenously provided growth factors.[15,16] We therefore considered the possibility that CD8$^+$ MHC class II-specific T cells in MHC class II-deficient mice could be missed because of the dominance of class I-specific clones. In order to avoid stimulation by allogeneic MHC class I molecules during the primary MLC, we used β_2M and thereby class I-deficient spleen cells as stimulators. Although these mice bear the same H-2^b haplotype as the class II^0 mice, the mutation of the Aβ gene renders them allogeneic with respect to class II. We compared the class II-induced cytotoxic responses of CD8$^+$ spleen cells of class II^0 and B10.BR mice. CD4-depleted B10.BR spleen cells were used as a control. Heterozygous, class II-expressing responder mice could not be used for this purpose because they share the MHC class II haplotype with class I-deficient mice. CD4-depleted spleen cells from class II^0 and from B10.BR mice stimulated with the completely allogeneic DBA/2 spleen cells developed high cytotoxic activities against P815 target cells (expressing K^d, D^d and L^d) (Fig. 2). However, CD4-depleted spleen cells from class II^0 mice stimulated with class I-deficient, class

II-expressing cells developed significantly ($P < 0.01$) higher cytotoxic activities against class II-expressing target cells than effectors from control mice. No cytotoxicity was detected with Con A blasts from spleen cells of class I-deficient or of normal mice, suggesting that the cytotoxicity was indeed class II-specific (Fig. 2). Cytolytic activities also developed against class I- and class II-expressing LPS blasts, but not against only class I-expressing Con A blasts of 129 and B6 spleen cells (data not shown). Flow cytometric analysis of the effector populations revealed that more than 98% of the Thy-1.2$^+$ cells were CD8$^+$ in all of the cultures tested (data not shown). Less than 1% were $\gamma\delta$ TCR$^+$ or CD4$^+$ T cells. These results were obtained in three independent experiments.

CD4$^-$ 8$^+$ T cells from mice with normal class II expression do not develop significant levels of class II-specific response in primary MLC

Since the control, class II-expressing and class II^0 mice might also differ in other genes beside MHC class II, we analysed several additional mouse strains for their capacity to develop, under the same culture conditions, class II-specific, CD8$^+$-mediated cytotoxic responses. All the responders were depleted of CD4$^+$ T cells and stimulated with spleen cells allogeneic only for the class II region. The following combinations of mouse strains were tested: B10.D2(R107) against B10.A(5R) (Fig. 3a), B10.A(4R) against B10.A(2R) (Fig. 3b), B10.GD against B10.HTG (Fig. 3c) and B6 against bm12 (Fig. 3d). In all cases, DBA/2 spleen cells were used as control, class I allogeneic stimulators, and P815 cells as class I-expressing targets. As expected, in all the experiments, CD4$^-$ 8$^+$ T cells developed high class I-specific cytotoxic responses (Fig. 3), but no class II-specific cytotoxicity was observed in these four combinations.

DISCUSSION

The data presented in this report provide evidence for an increased number of class II-specific, cytotoxic T cells in the CD4$^-$ 8$^+$ T-cell subset of class II-deficient mice. Upon stimulation with spleen cells allogeneic for MHC class II molecules, only effectors from class II^0 mice developed significant cytotoxicity against class II-expressing targets.

The large majority of CD4$^-$ 8$^+$ T cells bear TCR with specificities for self-class I molecules and antigen and/or for allogeneic class I molecules. However, a small number of CD4$^-$ 8$^+$ T cells specifically recognize allogeneic class II molecules.[14,17] It is not clear how T cells with such specificities develop. One possibility is that they are positively selected for class I, but cross-react in the periphery with class II molecules.[9,18] It has been suggested that the number of T cells bearing such cross-reactive TCR is rather low because most of them are negatively selected for during their ontology on class II molecules,[23] which they recognize with high affinity [9]. This possibility seems even more likely as the crystal structures of class I[19] and class II[20] molecules reveal many similarities. Another possible explanation for the existence of such cells could be that they are positively selected for by class II molecules in the thymus but, in a stochastic process that down-regulates CD4, develop into CD4$^-$ 8$^+$ T cells.[21,22] If the first explanation is correct, the number of CD4$^-$ 8$^+$ class II-reactive T cells should be increased in class II^0 mice. On the other hand, such T cells should be undetectable in class II^0 mice if the second hypothesis is correct. Since CD4$^-$ 8$^+$ class II-specific cells were increased in class II^0 mice, we conclude that they indeed are positively selected for by self-class I molecules and survive because of the lack of negative selection by class II molecules.

In our experiments no significant numbers of CD4$^-$ 8$^+$ cytotoxic T cells specific for class II molecules could be detected when responder cultures were stimulated with spleen cells expressing both allogeneic class I and class II molecules. We considered the possiblility that the class II-specific CD4$^-$ 8$^+$ T cells are overgrown by class I-specific T cells.[13–15] Indeed, when responders were stimulated with spleen cells of class I-deficient mice, effector cells from class II^0 mice but not from class II-expressing mice developed cytotoxic activities against MHC class II$^+$ target cells.To exclude an influence of the particular MHC haplotypes of the mouse strains used in these experiments, we analysed cytolytic T-cell responses of CD4$^-$8$^+$ T cells from four additional mouse strains. No significant, class II-specific cytotoxicity was detectable in these T-cell populations. We therefore concluded that the number of CD4$^-$ 8$^+$ T cells with specificity for class II was increased in class II^0 mice because of the lack of negative selection on class II molecules during ontology of the T cells. This effect was independent from the particular allotype of the MHC molecules involved.

The original studies with class II^0 mice described an overall increase in numbers of CD8$^+$ T cells in comparison to their class II-expressing littermates.[3,4] We observed the same in the mice used in our experiments. Usually, class II^0 mice had around 30% more CD8$^+$ T cells in the starting populations, i.e. after elimination of the CD4$^+$ subset. However, this increase in the absolute number of CD8$^+$ cells did not influence the results obtained in these studies. ^{51}Cr-release assays were always performed with the same number of effector cells, and class II-specific cytotoxicity of CD4$^-$8$^+$ T cells was only observed with cells from class II^0 mice. Moreover, increasing the number of responder cells in the primary MLC did not alter the results (data not shown). The effectors in the cytotoxicity assays were indeed of the CD4$^-$ 8$^+$ phenotype: More that 98% of all Thy-1.2$^+$ T cells were CD4$^-$ 8$^+$, and all CD8$^+$ T cells were TCR$\alpha\beta^+$ cells. Less than 1% of the cells were CD4$^+$ 8$^-$ or TCR$\gamma\delta^+$ cells, making them unlikely effectors responsible for the cytolysis observed in the described experiments. The targets included LPS and Con A blasts from both class I-negative mice (β_2M and class I-deficient) and class I-expressing mice bearing the H-2^b haplotype and of 129 or B6 background. Effector cells from class II^0 but not from class II-expressing mice lysed the LPS blasts. Con A blasts were not recognized. These results strongly suggest that the target molecule was indeed the MHC class II molecule A^b.

Based on these results, we suggest that CD4$^-$ 8$^+$ T cells with specificity for class II molecules develop upon engagement of their TCR with class I rather than class II molecules. The same conclusion was derived by Mizuochi *et al.*[18] from their analysis of CD4$^-$ 8$^+$ T cells in mice neonatally treated with anti-class II mAb. In contrast, Kirberg *et al.*[24] suggested that T cells expressing class II-restricted, transgenic TCR develop into CD8$^+$ cells after engagement with class II molecules. However, they found that these cells also needed class I molecules for

their development. The $CD4^-8^+$ class II-specific T cells that we detected in the periphery of class II^0 mice did not require class II molecules for induction of cytolytic responses. The increase of their number was almost certainly due to the lack of negative selection of developing thymocytes on class II molecules in class II^0 mice.

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